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Chondrocyte morphology in stiff and soft agarose gels and the influence of foetal calf serum

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Abstract

Changes to chondrocyte volume/morphology may have deleterious effects on extracellular matrix (ECM) metabolism potentially leading to cartilage deterioration and osteoarthritis (OA). The factors controlling chondrocyte properties are poorly understood however pericellular matrix (PCM) weakening may be involved. We have studied the density, volume, morphology and clustering of cultured bovine articular chondrocytes within stiff (2%w/v) and soft (0.2%w/v) 3-dimensional agarose gels. Gels with encapsulated chondrocytes were cultured in Dulbecco's Modified Eagle's Medium (DMEM; foetal calf serum (FCS) 1-10%;380mOsm) for up to 7d. Chondrocytes were fluorescently-labelled after 1, 3 and 7d with 5-chloromethylfluorescein-diacetate (CMFDA) and propidium iodide (PI) or 1,5-bis{[2-(dimethylamino)ethyl]amino}-4,8-dihydroxyanthracene-9,10-dione (DRAQ5) to identify cytoplasmic space or DNA and imaged by confocal laser scanning microscopy (CLSM). Chondrocyte density, volume, morphology and clustering were quantified using Volocity™ software. In stiff gels after 7d with 10% FCS, chondrocyte density remained unaffected and morphology was relatively normal with occasional cytoplasmic processes. However in soft gels by day 1, chondrocyte volume increased ($P=0.0058$) and by day 7, density increased ($P=0.0080$), along with the percentage of chondrocytes of abnormal morphology ($P<0.0001$) and enhanced clustering ($P<0.05$), compared to stiff gels. FCS exacerbated changes to density ($P<0.01$), abnormal morphology ($P<0.001$) and clustering ($P<0.01$) compared to lower concentrations at the same gel strength. Reduced gel stiffness and/or increased FCS concentrations promoted chondrocyte proliferation and clustering, increased cell volume, and stimulated abnormal morphology, producing similar changes to those occurring in OA. The increased penetration of factors in FCS into soft gels may be important in the development of these abnormal chondrocyte properties.

Introduction

Chondrocytes are exclusively responsible for the maintenance of articular cartilage through a balance between the synthesis and degradation of extracellular matrix (ECM) macromolecules. In osteoarthritis (OA) due to an imbalance in chondrocyte matrix metabolism, normal turnover is deleteriously affected thereby leading to cartilage degeneration. These disturbances include loss of proteoglycans and disorganization/loosening of the collagen network which begins near the cartilage surface (Hollander et al., 1995) leading to softening/swelling of the cartilage, reduced resilience and eventually its failure (Buckwalter and Mankin, 1997). The mechanisms underlying the initiation and progression of the degenerative process are complex and unclear, but changes to ECM metabolism by chondrocytes are recognised as a key component of OA.

A clue to some of early events in the development of OA have come from studies which have identified changes to the normal morphology of chondrocytes within macroscopically normal cartilage and which appear to become exacerbated as OA progresses. This suggests that they might be involved either directly or indirectly in the process of cartilage failure. Thus the normal elliptical/spherical morphology of articular chondrocytes becomes disturbed as there are increases in cell volume (Bush and Hall, 2003), alterations to chondrocyte shape (Kouri et al., 1998; Bush and Hall, 2003; Holloway et al., 2004) and as OA develops, cell clustering (Lotz et al., 2010). These observations are of significance because chondrocyte matrix metabolism is strongly influenced by cell volume (Urban et al., 1993), morphology/cytoskeletal structure (Benya and Shaffer, 1982; Brown and Benya, 1988; Woods et al., 2007; Murray et al., 2010) and chondrocyte clustering (Lotz et al., 2010). Therefore, an understanding of these changes to chondrocytes in the initial and developing stages of OA could be important for limiting deleterious changes to matrix metabolism.

Modelling these changes to chondrocytes in a controlled way in an attempt to follow the alterations to chondrocyte matrix metabolism associated with OA initiation and development is exceptionally difficult. However, the culture of chondrocytes in biocompatible three-dimensional (3D) structures made of natural or synthetic hydrogels (e.g. alginate, agarose) has given important fundamental information on some of the factors controlling the synthesis/breakdown of the ECM (Aydelotte et al., 1986). Such hydrogels promote the chondrocytic phenotype in culture by maintaining their morphology (Benya and Shaffer, 1982; Häuselmann et al., 1992) and the synthesis of a mechanically-viable proteoglycan-collagen matrix (Buschmann et al., 1992; Mauck et al., 2000) and strongly resembles the composition of their native environment including deposition of collagen type VI in the pericellular matrix (PCM) (Chang and Poole, 1996; Chang et al., 1997).

The relatively stiff PCM links chondrocytes to their extracellular (inter-territorial matrix) environment by components which provide crucial information about the prevailing biochemical and biomechanical environment. The PCM is primarily characterized by the presence of type VI collagen but also possesses hyaluronan and high concentrations of proteoglycans, including aggrecan, decorin, as well as fibronectin, type IX collagen (Poole, 1997) and perlecan (Wilusz et al., 2012). The process of ‘stiffness sensing’ is thought to be achieved through the cytoskeleton and focal adhesions and has been shown to be important for cellular mechanotransduction in a range of other cell types (e.g. (Engler et al., 2006)). Loss of PCM stiffness appears to be associated with development of OA (Alexopoulos et al., 2003) and could be the basis for the abnormal mechanotransduction associated with this disorder (Wilusz et al., 2012). Varying the concentration of agarose for 3D culture of articular chondrocytes has been used to approximate the normal stiff PCM of cartilage ($\geq 4.5\%$ agarose) and the softened PCM of OA cartilage ($\leq 3.0\%$ agarose) (Jutila et al., 2015). It is known from various studies that gel strength influences the compressive properties of gel constructs and

also matrix synthesis by encapsulated cells including chondrocytes (Aydelotte et al., 1986; Balgude et al., 2001; Ng et al., 2009; Schuh et al., 2011). Damage to the ECM in OA may also lead to enhanced access of growth factors to chondrocytes thereby leading to chondrocyte hypertrophy-like changes (Tchetina et al., 2007) and cluster formation (van Susante et al., 2000). Changing the stiffness of gel constructs might therefore reveal properties of the encapsulated chondrocytes that have parallels to the changes that occur in OA when PCM stiffness declines.

During preliminary experiments investigating the fine morphology of fluorescently-labelled chondrocytes using confocal scanning laser microscopy (CLSM) (Karim and Hall, 2013), we noted that in relatively stiff agarose gels (2% w/v) the cells were typically rounded/spheroidal and similar to their morphology *in situ*. However there were dramatic changes to chondrocyte density, shape, volume and clustering during culture in soft gels (0.2%) particularly with increasing concentrations of foetal calf serum (FCS). In the present study we have developed this 3-dimensional (3D) model and used quantitative CLSM imaging to compare the effect of chondrocyte culture in stiff or soft agarose constructs at various FCS concentrations on chondrocyte properties (cell density, volume, clusters, morphology). Of particular interest was that some of the changes observed in chondrocyte properties in this model (e.g. increased density, cluster formation, cell swelling, abnormal morphology) appeared to recapitulate those occurring in OA cartilage. In addition, the marked alterations to chondrocyte properties could be suppressed by increasing gel strength and/or decreasing FCS concentration.

Materials and Methods

Biochemicals and Solutions

Biochemicals were from Invitrogen Ltd., (Paisley, UK) or Sigma-Aldrich (Poole, UK) unless stated otherwise. The standard culture medium used throughout was Dulbecco's Modified Eagle's Medium (DMEM) with NaCl added to increase osmolarity to within the range experienced by chondrocytes *in situ* (380mOsm; (Urban et al., 1993)), 10% foetal calf serum (FCS, not heat inactivated), 50µg/ml ascorbic acid and penicillin/streptomycin (100U/ml;100µg/ml respectively). For some experiments, the composition of the culture medium was altered by FCS addition (1%, 2%, 5% and 10%; 380mOsm for all solutions). The viability probe, 5-chloromethyl-fluorescein diacetate (CMFDA), and the nucleic acid dye propidium iodide (PI), were prepared as described (Amin et al., 2008). DRAQ5 (DNA probe) was purchased as a 5mM stock solution (Invitrogen Ltd.). DRAQ5 and PI can both bind to DNA but differ in their ability to label nuclei as PI is membrane-impermeable and binds to nuclei of dead cells only, whereas DRAQ5 is membrane-permeable and can stain nuclei of both live and fixed cells. PI and DRAQ5 were therefore used for determining cell death and counting of chondrocytes in clusters, respectively. Formaldehyde solution (4% v/v in saline; pH7.3) was from Fisher Scientific (Leicestershire, UK). Other biochemicals used for cell isolation and culture were collagenase Type I, (255units/mg) and UltraPure™ low melting point (LMP) agarose powder, both from Invitrogen Ltd.

Cartilage removal and isolation of chondrocytes

Fresh bovine metacarpal-phalangeal joints of 3yr. old cows obtained from a local abattoir, were dissected under aseptic conditions. Only non-degenerate joints with normal healthy cartilage were selected and full depth cartilage fragments were removed from load-bearing areas of the joint by scalpel. Bovine subchondral bone is very hard and virtually impossible to cut by scalpel. Thus, it is unlikely that any bone cells would be present during

isolation, however chondrocytes would be isolated from all cartilage zones and therefore a heterogeneous cell population would be present during culture. Fragments were washed twice with DMEM (380mOsm) and then digested with DMEM-collagenase (0.8mg/ml) for approx. 18hrs at 37°C (380mOsm; pH 7.4) to release the chondrocytes. The partially-digested matrix was rinse-filtered through a tea strainer and then a nylon cloth filter (pore size 200µm; Cadisch Ltd., London, UK) and the filtrate centrifuged (800xg; 10min; 21°C). The cell pellet was re-suspended in DMEM and only samples with >95% viable chondrocytes as determined by Trypan blue exclusion were used for experiments.

Chondrocyte culture in gels of varying strengths and fluorescent labelling

Stock agarose gel (4% w/v) was prepared by mixing LMP powder in phosphate-buffered saline (PBS; 37°C) and maintained at approx. 37°C throughout. Chondrocyte suspensions with agarose at 2% or 0.2% w/v defined as ‘stiff’ or ‘soft’ respectively, were prepared in wells and then placed on ice for 2hrs to allow the gels to set. Cell density in the gels was approx. 15×10^6 cells/ml similar to seeding densities previously described (Buschmann et al., 1995). Gels were incubated with standard DMEM (380mOsm; 37°C; 5% CO₂; pH 7.4) for up to 7 days, with medium changed on alternate days. At days 0 and 1 and during the last hour of culture, gels were incubated with CMFDA (12.5µM) and PI (5µM) prepared in PBS to identify live or dead cells respectively as described (Amin et al., 2008). However because of chondrocyte proliferation within clusters and the difficulty in identifying individual chondrocytes, at days 3 and 7, in addition to CMFDA, DRAQ5 (18µM) prepared in PBS (Martin et al., 2005) was used instead of PI. After incubation with fluorescent dyes, gels were then washed in PBS, fixed in 4% formaldehyde (30mins) and mounted in small cell culture dishes (Cellstar®; 35x10mm) and imaged by CLSM.

Confocal Laser Scanning Microscopy (CLSM)

A Zeiss Axioskop LSM510 upright CLSM (Zeiss U.K., Welwyn Garden City, U.K.) was used with E_x of 488nm, 543nm and 633nm to excite the fluorophores CMFDA, PI or DRAQ5 respectively and the emission measured through a band pass filter of 505-530nm for CMFDA and a long pass filter of 650nm for PI or DRAQ5. Fluorescently-labelled chondrocytes were imaged in the axial plane using high power (x40; NA=0.8; dipping water (DW) lenses to obtain details of the fine morphology of individual cells. The complete set of z stack images typically to a depth of 100 μ m, comprised single confocal sections at intervals of 1 μ m and frame size of 1024x1024 pixels with a scanning speed of 9 frames/sec. Optical sections were converted into three-dimensional (3D) reconstructions using VolocityTM (Improvision, Coventry, UK) software (Amin et al., 2008).

Morphological analysis of cultured chondrocytes

Quantitative data regarding cell density, volume and 3D morphology of cultured chondrocytes were obtained from the CLSM (x40 DW objective) images using VolocityTM software. Regions of interest (ROIs) with dimensions of x , y , z 230, 230 and 100 μ m respectively were applied to all images and data acquired. The number of cells was counted in the specified ROIs and cell density calculated as the number of cells/mm³ $\times 10^3$ by the formula; cell density = total no. of cells labelled with CMFDA, and PI or DRAQ5 / volume of gel imaged (μ m³). The volume of chondrocytes was determined only on morphologically-normal cells (i.e. cells with no cytoplasmic processes), on a cell-by-cell basis using a measurement protocol on VolocityTM with a threshold segmentation method to identify the cell edge. Volume measurements were calibrated using fluorescent latex beads (10.16 μ m diam.; 549 μ m³; FluoresbriteTM, Polyscience Inc., Warrington, UK (Bush and Hall, 2001)), imaged under conditions identical to those used for cultured chondrocytes in gels. The measuring protocol determined single cells as separate items but clusters were identified as a whole unit because

segmentation of cells in a cluster was not possible as the membranes of cells were frequently touching. Clusters were defined as ≥ 3 chondrocytes together as a unit because normal bovine cartilage chondrocytes may occur in pairs (Sasazaki et al., 2008). The volume of clusters was determined by the same methodology as for individual cells and the number of cells in the clusters was counted by eye as DRAQ5-labelled nuclei. Only living cells labelled with both CMFDA (to confirm membrane integrity) and DRAQ5 were assessed. DRAQ5 labelling in the absence of surrounding CMFDA fluorescence was taken to indicate the presence of a dead chondrocyte. Over the 7 day period, cell viability when cultured in either stiff or soft agarose gels (with FCS) was $>95\%$ of the cell population (data not shown). The images of chondrocytes in Figs. 1 and 6 show the fluorescence emitted by both CMFDA (green) and DRAQ5 (red) within living chondrocytes. While the DRAQ5 signal was similar between chondrocytes, CMFDA fluorescence depended on chondrocyte volume (Karim & Hall, 2016) and this probably accounts for various cell colours observed (Fig. 1A & Fig. 6).

The volume of individual cells in a cluster was calculated as: volume of individual cells in cluster = (total volume of cluster in μm^3 /total number of cells in cluster). The percentage of chondrocytes forming clusters was calculated as: % of chondrocytes forming clusters = $100 \times (\text{number of DRAQ5-labelled cells forming clusters}/\text{total number of cells}) \%$.

Chondrocyte morphology was assessed in 3D using VolocityTM. Chondrocytes which were spheroidal/elliptical and having no cytoplasmic processes as visualised using the methods described above were considered as having ‘normal’ morphology however those with non-spheroidal shapes and having CMFDA-labelled cytoplasmic processes emanating from the cell body were considered ‘abnormal’ (Bush and Hall, 2003). The length of cytoplasmic processes was determined in 3D by eye, by tracing the processes and measuring the overall length from its origin at the cell body, to the end of the process. Cytoplasmic processes were categorised as ‘short’ ($\leq 29.9 \mu\text{m}$), ‘medium’ (30 to $59.9 \mu\text{m}$) or ‘long’ ($\geq 60 \mu\text{m}$). The percentage of abnormal

chondrocytes was calculated as: % abnormal chondrocytes = $100 \times (\text{number of abnormal cells} / \text{total number of normal and abnormal cells}) \%$.

Data presentation and analysis of results

Data were presented as mean \pm SEM with N representing the number of different animals and n' the number of cells for each experimental condition. Statistical tests and graphs were generated using Graph Pad Prism 6 (GraphPad Inc., USA). Unpaired, two-tailed, Student's t-tests were used to compare means within groups. One-way analysis of variance (one-way ANOVA) followed by Tukey's multiple comparison *post-hoc* test was used to compare data between groups. A significant difference was accepted when $P < 0.05$. * indicated a significant difference according to unpaired Student's t-tests within groups, # indicated a significant difference according to one-way ANOVA between the groups compared. Single, double and triple symbols showed the level of significance for $P < 0.05$, 0.01 and 0.001 respectively.

Results

(A) Effect of agarose gel strength on the density and morphology of bovine chondrocytes cultured in 3D agarose gels with 10% FCS

Fig. 1(A) shows the overall changes to chondrocyte density and morphology following culture with 10% FCS in stiff (2%) or soft (0.2%) agarose gels. Over 7 days of culture in stiff gels, chondrocyte morphology remained relatively normal with small cytoplasmic processes being observed only very occasionally (Fig. 1A(a-c)) but with no significant change to chondrocyte density (Fig 1B). However in soft agarose gels, abnormal chondrocyte morphology was evident by day 3, with more cells exhibiting cytoplasmic processes by day 7 (Fig. 1A(f)). Chondrocyte density increased significantly by approx. 1.7-fold by day 7 (Fig. 1B) compared to cells in stiff gels, with cell clusters being clearly evident (Fig. 1A(f)).

Chondrocyte morphology remained relatively 'normal' (i.e. spheroidal/elliptical with no cytoplasmic processes) at day 1 in both gel strengths (Fig. 1A(a,d)). However, the volume of isolated chondrocytes increased significantly by day 1 when cultured in soft gels as compared to stiff gel cultures (Fig. 2(a)). In the stiff and soft gels, the chondrocyte volume ranged from $263\mu\text{m}^3$ to $2413\mu\text{m}^3$ and from $331\mu\text{m}^3$ to $3547\mu\text{m}^3$ respectively, with highest frequencies of chondrocyte volumes in the stiff and soft gels in the range of $700\text{-}800\mu\text{m}^3$ and $900\text{-}1100\mu\text{m}^3$ respectively (data not shown).

A quantitative assessment of chondrocyte morphology was performed on cultures over 3 days of culture (Fig. 2(b)). Although there was an increase in the volume of morphologically-normal chondrocytes at day 1 (Fig. 2(a)) there was no change to the % of cells with processes (Fig. 2(b)). This therefore suggested that the volume increase may precede the development of cytoplasmic processes. However, by day 3 in soft gels, the percentage of chondrocytes with processes increased significantly by approx. 10-fold, (to approx. 30%) whereas there was no change for cells in the stiff gels (approx. 3%; Fig. 2(b)). There were between one and two processes/cell for the very small % of chondrocytes in stiff gels (Fig. 2(c)) whereas in the soft gels, the chondrocytes possessed between two and three processes/cell although this did not reach the level of significance (Fig. 2 (c)). Moreover, in the soft gels, the production of small processes was preferred as the percentage of chondrocytes having short processes was significantly higher than those medium and long processes (Fig. 2(d); $P<0.001$). In contrast, very few chondrocytes (approx. 3%) in stiff gels produced cytoplasmic processes of any length. At day 3, the extent of cluster formation was not significantly different between the gel strengths (data not shown) suggesting that cytoplasmic processes were produced before chondrocyte clustering occurred.

The changes to chondrocyte properties observed over 3 days in culture were greatly increased by day 7. The development of cytoplasmic processes was now evident in stiff gels

($18\pm 1\%$ of the cell population) but there were almost 3-fold more abnormal chondrocytes in soft gels (Fig. 3(a); $67\pm 1\%$; $P<0.0001$). There was now a significantly higher number of processes per cell in soft gels (Fig. 3(b); $P=0.0002$) compared to day 3 (Fig 2(c)). An analysis of the length of these processes demonstrated the presence of only small and medium sized categories in stiff gels, but all three groups of lengths were present in soft gel cultures with a significant difference in the average length of these categories ($P<0.001$; (Fig. 3(c)). By day 7, a significantly higher percentage of chondrocytes in soft gels demonstrated cytoplasmic processes for small and medium length categories compared to stiff gels (Fig. 3(d); $P<0.0001$; $P=0.0017$ for small and medium respectively) and no difference observed for long processes. The majority of ‘abnormal’ chondrocytes observed in soft and stiff gels demonstrated the presence of small cytoplasmic processes ($67\pm 3.2\%$ and $21\pm 5\%$; Fig. 3(d)). Medium and long processes were also occasionally observed in soft gels with similar processes only rarely found in stiff gels (Fig. 3(d)). Thus, broadly similar changes to chondrocyte morphology occurred in both strengths of gels although the extent of this was far more marked in the soft (0.2%) gels.

By day 7, chondrocyte clustering was an obvious morphological feature routinely observed particularly in soft gels but also occasionally in stiff gels (Fig. 1A(f)). The number of clusters present, and the number of cells per cluster in the soft gels, were significantly higher compared to those in stiff gels (Fig. 4(a,b); $P<0.05$; $P=0.002$ respectively). In the soft gel cultures, a significantly higher percentage of chondrocytes formed clusters ($76\pm 5\%$ compared to $41\pm 5\%$ in stiff gels, Fig. 4(c)). The volume of clusters was significantly greater for chondrocytes cultured in soft gels (Fig. 4(d)), however the volume of individual chondrocytes in a cluster showed no significant difference between the gel strengths ($725\pm 39\mu\text{m}^3$ vs $736\pm 42\mu\text{m}^3$ from $[N(n')]=[6(313)$ and $6(525)]$ in stiff and soft gels respectively).

(B) Effect of increasing concentrations of FCS on properties of bovine chondrocytes cultured in stiff and soft 3D agarose gels

The previous results suggested that the morphological changes to chondrocytes and their sequence which occurred during culture with 10% FCS in stiff and soft gels were very similar, but were markedly accelerated in the latter gels. This raised the possibility that the penetration of growth/mitogenic factors in FCS was greater in the soft gels. The effects of FCS concentration (1-10%) were therefore tested on chondrocytes in stiff and soft agarose culture and cell density, volume and morphology (clusters/cytoplasmic processes) assessed. Serum-free (0% FCS) was not utilised as a culture condition because FCS-supplemented medium is required for chondrocyte metabolism and maintenance of phenotype as previously reported (Glaser and Conrad, 1984). The volume of chondrocytes with normal morphology in soft gels at day 1 was significantly greater compared to those in stiff gels in the presence of 2%, 5% and 10% FCS (Fig. 5; $P < 0.05$ for the three higher concentrations of FCS) but not with a low (1%) concentration. Measurements of cell volume were only performed at day 1 because as culture progressed beyond this, complex changes to chondrocyte morphology (clusters/cytoplasmic processes) rendered volume measurements difficult and cell boundaries were frequently touching making the discrimination of individual cells potentially inaccurate. The analysis of chondrocyte density, morphology and clustering was performed only at day 7 because after this, huge clusters of abnormal cells were formed (data not shown) and measurements on single chondrocytes were not possible. By day 7, the density and morphology of chondrocytes cultured in soft gels had altered markedly with increasing concentrations of FCS (Fig. 6(e-h)) whereas relatively few changes to chondrocyte properties were observed in stiff gels even at 10% FCS (Fig. 6(a-d)).

In the soft gel cultures in the presence of increased concentrations of FCS (5% and 10%), a significantly higher percentage of chondrocytes with processes were present compared to stiff

gels (Fig. 7(a)). Chondrocytes cultured in soft gels with 10% FCS had a significantly higher percentage of abnormal cells compared to the lower concentrations (1%, 2% and 5%) of FCS (Fig. 7(a); $P<0.001$). Additionally, soft gels cultured with 2% and 5% FCS, had a significantly higher percentage of cells with processes as compared to those with 1% FCS in the same gel strength ($P<0.01$ and $P<0.05$ respectively). However in stiff gels, the percentage of cells with processes remained small (approx. 10%) and unaffected by increasing concentrations of FCS (Fig. 7(a)).

The number of processes per cell was significantly higher in soft gels with 10% FCS as compared to stiff gels ($P=0.0002$) and also in comparison to all the lower concentrations (1%, 2% and 5%) of FCS in the same gel strength (Fig. 7(b); $P<0.001$; $P<0.001$ and $P<0.01$ respectively). Similarly in soft gels with 5% FCS, the number of processes per cell was significantly higher as compared to stiff gels ($P<0.0001$) and also in comparison to the lower concentrations (1% and 2%) of FCS in the same gel strength (Fig. 7(b); $P<0.001$). However, in the stiff gel cultures, for the abnormal chondrocytes which represented only a small fraction of the total cell population (approx. 10%) the number of processes per cell remained unaffected with the increasing concentrations of FCS.

Cell density remained unaffected in the stiff gel cultures with increasing concentrations of FCS (Fig. 8(a); $P>0.05$ by ANOVA). However, in the soft gels, chondrocyte density increased significantly in the presence of 10% FCS as compared to 1% and 2% FCS (Fig. 8a; $P<0.05$) suggesting chondrocyte proliferation with increasing concentration of FCS.

Clustering was greater in soft gel cultures with increasing FCS concentrations. There was a significantly higher number in soft gels with 5% and 10% FCS as compared to stiff gels ($P=0.04$ in both conditions) but was not significantly different at 1% and 2% FCS between gel strengths (Fig. 8(b)). Additionally, in soft gels in the presence of higher concentrations of FCS (5% and 10%), a significantly higher number of clusters were present as compared to 1% FCS

(Fig. 8(b); $P<0.01$). The number of clusters in stiff gels appeared to increase with FCS concentration, but this was not significant (Fig. 8(b); $P=0.14$ by ANOVA).

In the presence of 10% FCS, the number of cells per cluster was significantly higher in soft gels as compared to stiff gels ($P=0.01$) and also in comparison to the lower concentrations (1%, 2% and 5%) of FCS at the same gel strength (Fig. 8(c); $P<0.05$, $P<0.001$ and $P<0.01$ respectively). However, the number of cells per cluster remained unaffected in stiff gels with increasing concentrations of FCS ($P>0.05$). In the soft gels with 10% FCS, a high percentage of cells was present in clusters as compared to stiff gels (Fig. 8(d); $80\pm7\%$ vs $53\pm11\%$; $P=0.01$), compared to 1% and 2% FCS in the same gel strength ($P<0.001$; $P<0.01$ respectively) and also in the soft gels with 5% FCS as compared to 1% FCS ($P<0.05$). However, although it appeared that in stiff gels there was an increase in the percentage of chondrocytes present in clusters with rising FCS concentration, this did not reach significance (Fig. 8(d); $P=0.43$).

The overall volume of individual chondrocyte clusters formed in soft gels in the presence of 10% FCS was significantly higher than in the stiff gels ($P=0.009$) and also in comparison to 1% and 2% FCS in the same gel strength (Fig. 8(e); $P<0.05$ and $P<0.01$ respectively). However, the volume of clusters in stiff gels remained unaffected in all the four concentrations of FCS ($P>0.05$). Furthermore, the volume of single morphologically-normal cells in clusters remained unaffected in both stiff and soft gels with increasing concentrations of FCS (Fig. 8(f)). Increasing concentrations of FCS therefore strongly accelerated cluster formation in soft gels but had only a limited effect in stiff gels.

These results demonstrated that the changes to chondrocytes properties in soft gels were markedly accelerated by raising the FCS concentration, whereas the effects on cells in stiff gels were broadly similar, although very limited.

Discussion.

Studying the detailed morphological properties of fluorescently-labelled *in situ* chondrocytes in stiff and soft 3D agarose gels in the presence/absence of FCS by quantitative CLSM imaging, revealed marked changes to cell density, volume, cell shape and clustering. This approach is relatively novel and revealed properties of chondrocytes in 3D culture which have not previously been described. While some of these observations might have been reported previously e.g. by histological methods or fluorescence microscopy, this was not available in the quantitative detail reported here. The present data showed that the culture of chondrocytes in FCS (10%) in soft (0.2%) gels increased cell volume, resulted in the development of abnormal morphology and cell clustering, while having only mild, but broadly similar effects when the cells were in stiff (2%) gels. Furthermore, increasing concentrations of FCS (1-10%) in soft gels accelerated these morphological changes, suggesting that gel strength, which potentially controlled the penetration of serum factors, may have profound effects on several key properties of chondrocytes maintained in culture. Of particular interest was the possibility that the changes to chondrocyte properties observed in soft agarose and/or during culture with FCS, recapitulated those occurring in chondrocytes during OA development.

The visualisation at high magnification of the fluorescently-labelled cytoplasmic space of cultured chondrocytes using CLSM and imaging software, made it possible to observe and quantify chondrocyte properties including their fine 3D morphology. Such features have been reported for chondrocytes within human and animal cartilage (e.g. Bush and Hall, 2003; Jones et al., 2005) however there have been relatively few comparable quantitative studies on chondrocytes in 3D culture. These classically have a rounded morphology and cell clustering has been reported under some conditions (Buschmann et al., 1992). However the presence of fine cytoplasmic processes has not to our knowledge been described in 3D cultured

chondrocytes although a variety of studies have reported cytoskeletal elements using fluorescence immunohistochemistry (e.g. Idowu et al., 2000). The paucity of these studies could be because the CLSM and imaging protocols used may not have had the required sensitivity, but also because preparation/fixation methods can cause chondrocyte shrinkage (e.g. Loqman et al., 2010) reducing the chances of detecting the fine processes. In the present work, chondrocytes were labelled with the thiol-reactive vital fluorescent dye, CMFDA. This is converted to the highly fluorescent and membrane-impermeant probe fluorescein, which fluoresces within the membrane-delimited intracellular space (Poole et al., 1996) thereby identifying cell morphology. It should be noted that the chondrocyte cilium which may be described as a cytoplasmic ‘process’, would not be observable using our methods as it requires specific fluorescent labelling (e.g. with tagged α -tubulin) and are very short (typically 1.5 μ m; Poole et al., 1985) compared to the cytoplasmic processes which are at least an order of magnitude greater (e.g. Fig. 1).

The accelerated development of abnormal chondrocyte morphology and cell clusters during culture in soft compared to stiff gels, could be due to the reduced gel stiffness/binding properties directly influencing chondrocytes. It has been suggested that cells, including chondrocytes, can ‘probe’ their environment and respond morphologically and metabolically by sensing the stiffness of the surrounding material (e.g. Engler et al., 2006; Schuh et al., 2010). Agarose is typically thought to be inert and does not contain any native ligands associated with cellular interactions with mammalian cells (Rowley et al., 1999; Sakai et al., 2007). We chose to use agarose alone as these gels containing Arg-Gly-Asp (RGD) adhesion molecules suppress the chondrocyte phenotype leading to reduced glycosaminoglycan production and increased collagen type I and decreased type II production (Schuh et al., 2011). Nevertheless, there is evidence that expansion of cytoplasmic processes (neurites) in dorsal root ganglion cells was inversely correlated to the mechanical stiffness of agarose gels in the range of 0.75-2.00%

(wt/vol) (Balgude et al., 2001). Thus it is possible that the increased volume of chondrocytes in soft gels at day 1 (Fig. 2(a)) and with elevated FCS concentrations (Fig. 5) raised the likelihood of membrane-bound cytoplasmic material extruding away from the chondrocyte cell body thereby forming a 'process'. Chondrocytes in stiff agarose gels have been shown to retain their differentiated 'chondrocytic' phenotype, and produce a more mechanically stable extracellular matrix (Buschmann et al., 1992) reducing the probability of cytoplasmic process formation. An alternative, but not un-related explanation for the development of chondrocyte processes and clusters, could be that factors present in FCS can penetrate the soft agarose gels more easily compared to the stiff gel thereby influencing cell behaviour. It is known that as the penetrability of serum (Johnson et al., 1996) and fluorescent dextrans (Leddy et al., 2004) is elevated with decreasing concentration of agarose, and thus the access of stimulatory morphogenic/growth factors in FCS (Fortier et al., 2011) is probably greater in soft, compared to stiff gels.

Chondrocyte clusters are a characteristic histological feature of human OA and of mechanical and chemical models of OA, and are often localised near fissures and clefts of upper cartilage layers (Marijnissen et al., 2002; Lotz et al., 2010). Proliferation of cartilage cells in human OA as measured by ³H-thymidine incorporation, has been identified as the principal mechanism for cluster formation (Lee et al., 1993). It is likely therefore that mitogenic factors present in FCS stimulate increased chondrocyte density and cluster formation (Lotz et al., 2010). The presence of chondrocyte clusters in agarose or alginate cultures have been described previously, and reported to be similar to those in intact human cartilage (Kolettas et al., 1995; Lin et al., 2009).

Cells in clusters in fibrillated OA cartilage show abnormal differentiation from the normal chondrocyte phenotype, including markers of hypertrophy e.g. collagen type X (von der Mark et al., 1992; Wang et al., 2004). The term 'hypertrophy' is usually applied to reflect

the similarity between these changes and those of cells in the growth plate. However there was no change in the volume of chondrocytes within clusters despite the marked increase in the number and overall volume of clusters (Fig. 8(c,e,f)). This is in contrast to the dramatic (typically 10-fold) increase in cell volume of ‘hypertrophic’ chondrocytes observed in the growth plate (Bush et al., 2008). Thus, while the term ‘hypertrophy’ might accurately reflect changes to chondrocyte matrix metabolism occurring in the growth plate and clusters, it should not be taken to indicate an increase in the size of 3D cultured chondrocytes in clusters as a volume increase did not occur (Fig. 8(f)). In order to clarify the mechanism of cluster formation, preliminary experiments were performed with BrdU labelling of cultured chondrocytes in both strengths of agarose gels using methods previously described (Tran-Khanh et al., 2010). The results indicated a marked difference in BrdU labelling pattern, with the majority of cells cultured in soft gels labelled as BrdU positive but with almost no BrdU positive staining detected amongst chondrocytes cultured in stiff gels (Karim, A. unpublished results). The positive BrdU labelling of chondrocytes in soft gels taken with increased chondrocyte density (Fig. 8(a)) suggested that proliferation was the key mechanism involved in cluster formation rather than aggregation of the existing chondrocyte population or cell swelling.

The present results demonstrated the marked effect of FCS on clustering of chondrocytes in soft gels, however the data on stiff gels while not reaching the level of significance (e.g. Fig. 8 (a,b,d)), did suggest that there was a similar, although delayed, pattern. It is probable that extending the incubation period for chondrocyte culture in stiff gels with 10% FCS could result in the development of clusters. It was also noted that for some properties of chondrocytes and clusters, increasing the FCS concentration from 5 to 10% produced significant changes (e.g. % of chondrocytes with processes (Fig. 7(a)); number of cells per cluster (Fig. 8(c)). This serum is known to have powerful mitogenic effects with FGF-2

considered to be a potent inducer of chondrocyte cluster formation (Lotz et al., 2010). The present model offers the opportunity of testing this and other components of FCS and synovial fluid for their ability to induce abnormal chondrocyte properties. We note that FCS added to 10% (v/v) is widely used for chondrocyte and cartilage culture however the changes to chondrocyte morphology and clustering reported here may indicate hitherto unforeseen and potentially unappreciated effects of FCS on chondrocyte matrix turnover and cell metabolism. Additionally, it has been reported that there are factors present in FCS which remove the DNA of dead chondrocytes probably as a result of DNAase activity (see Karim & Hall, 2016).

The morphological changes to chondrocytes observed in soft gels were strongly exacerbated when the concentration of FCS in the medium was increased. A wide variety of studies have demonstrated close relationships between chondrocyte morphology, cytoskeletal structure and the production of matrix components (e.g. Archer et al., 1982; Benya and Shaffer, 1982; Mallein-Gerin et al., 1991; Schnabel et al., 2002). Thus chondrocytes with a de-differentiated 'fibroblastic' morphology produce changes to gene expression/matrix synthesis leading to increased levels of collagen type I but reduced collagen type II and aggrecan production. In contrast, chondrocytes with a normal 'rounded' morphology produce cartilage-specific matrix macromolecules (Glowacki et al., 1983). Our study has not addressed the nature of the PCM synthesised by chondrocytes in stiff and soft gels. However, preliminary histochemical studies using Alcian blue suggested an increased staining intensity of a pericellular matrix (PCM) surrounding chondrocytes after 7 days when cultured in stiff compared to soft gels with 10% FCS, (Karim, A. unpublished). It would be of interest to utilise the 3D models described here with quantitative imaging/immunofluorescence methods, to study the composition/structural characteristics of the PCM, and the relationship between chondrocyte properties and gene expression analysis of matrix proteins and their degradative enzymes.

Although in our studies it was not possible to follow the changes to chondrocyte properties in the same population of cells in soft gel cultures over the 7 day culture period, it appeared that these followed a sequence, (a) by day 1, increased chondrocyte volume (Fig. 2(a), Fig 5), (b) by day 3, development of cytoplasmic processes (Fig. 2(b)), (c) by day 7, increased cell density (Fig. 1B; Fig. 8), and (d) cluster formation (Fig. 4; Fig. 8). It is noteworthy that these changes are similar to some of those observed in chondrocytes in human osteoarthritic (OA) cartilage (cell volume increase; (Bush and Hall, 2003), development of cytoplasmic processes (Bush and Hall, 2003; Murray et al., 2010), increased cell density/cluster formation (Lee et al., 1993; Lotz et al., 2010)). It is well known that during progression of OA, degenerative processes lead to several mechanical and physical changes such as matrix loosening (Aigner and McKenna, 2002), increased permeability, swelling (Wang et al., 2013) and decreased rigidity (Maroudas, 1976). It is possible that a significant contributor to the change in chondrocyte phenotype depends on the accessibility of factors in FCS or synovial fluid, and this is determined mainly by the agarose concentration in 3D culture and by matrix permeability in degenerate cartilage respectively. We note however that the diffusivity of tissue engineering constructs is generally higher than that of native cartilage (Leddy et al., 2004).

The 3D agarose constructs with the imaging methods described here offer the opportunity of quantitatively determining properties of chondrocytes which undergo some of the changes (abnormal morphology, cell swelling, cluster formation) identified in OA. The study of the relationship between individual chondrocyte morphology and their phenotype as defined by matrix synthesis/breakdown and the pathways involved, would be a worthwhile research direction, as they may model some of the changes to ECM metabolism which develop during OA.

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FIGURES

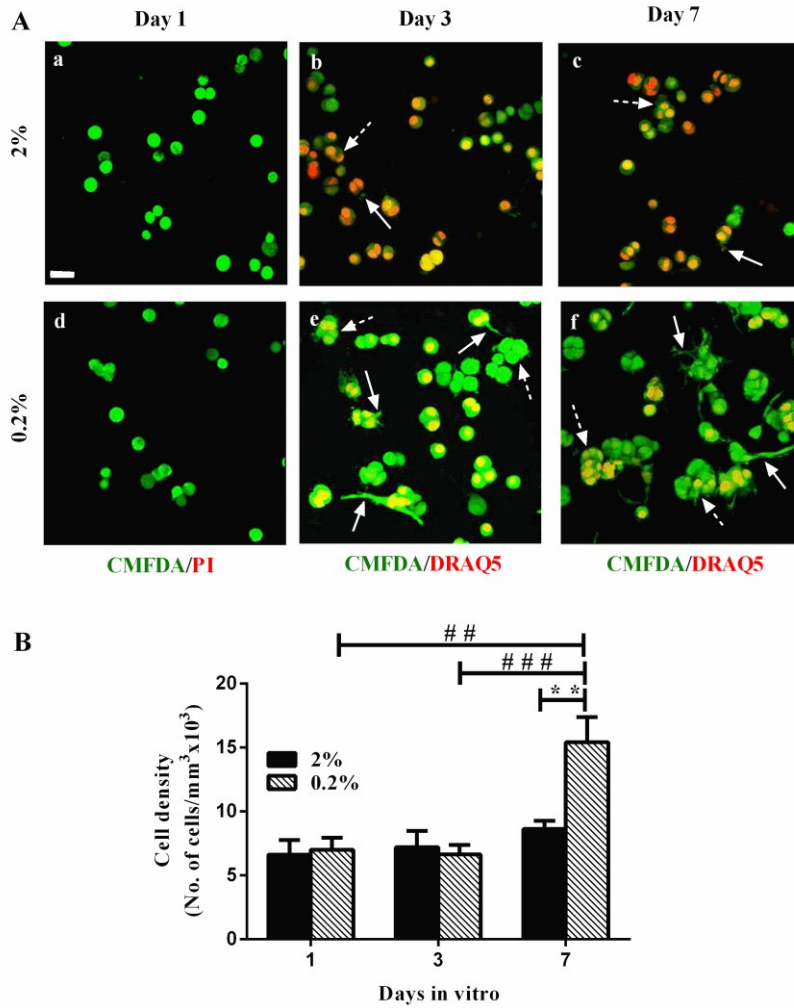


Fig. 1: Morphological changes to chondrocytes following culture in stiff (2%) or soft (0.2%) agarose gels in the presence of 10% FCS. (A) Axial CLSM high power (x40DW) reconstructed images of CMCDA/PI-labelled chondrocytes (cytoplasmic space of live cells/DNA labelling of dead cells respectively), (a, d) at day 1, and CMCDA/DRAQ5-labelled chondrocytes (corresponding to the cytoplasmic space of live cells and DNA labelling respectively), (b, e) at day 3 and (c, f) at day 7, cultured in stiff and soft agarose gels respectively. Note the range of colours of chondrocytes in panels (b, c, e, f) corresponding to the dual labelling of living cells with both green and red fluorescent dyes (see Materials and Methods for further details). Solid arrows indicate examples of cytoplasmic processes and broken arrows indicate examples of clusters. Scale bar for all panels = 25µm. (B) Chondrocyte density at various time points in stiff and soft gels. Data were from $N(n')=6(175, 228, 313)$ in stiff gels and $6(111, 491, 525)$ in soft gels at days 1, 3 and 7 respectively. * indicated a significant difference according to unpaired Student's T-test between stiff and soft gels, # indicated a significant difference according to a one-way ANOVA between soft gels. Double and triple symbols showed the level of significance for $P<0.01$ and 0.001 respectively.

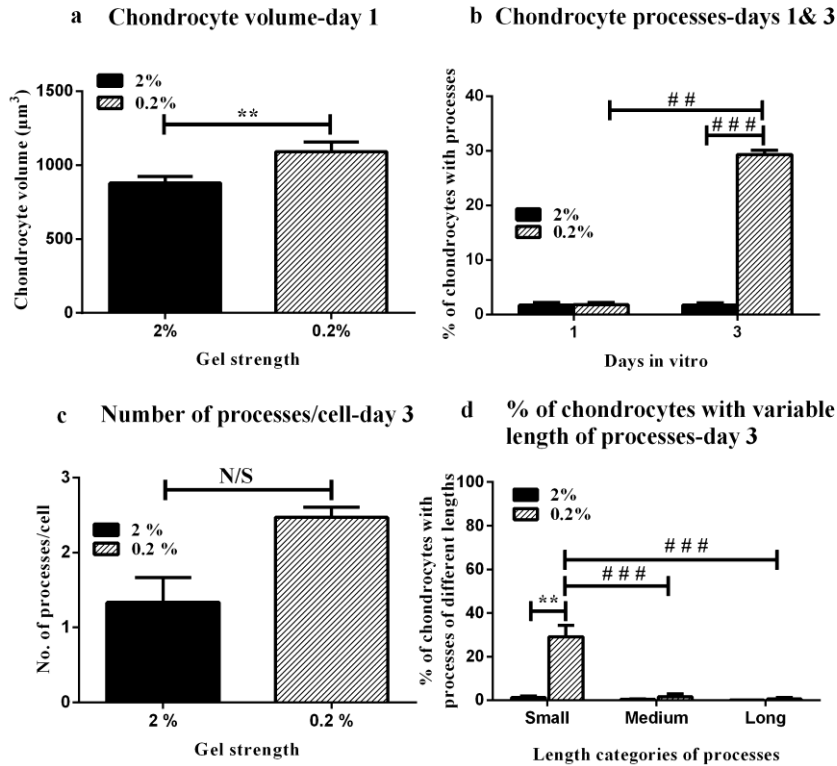


Fig. 2: Changes to chondrocyte volume and morphology during culture in stiff or soft agarose gels in the presence of 10% FCS. **(a)** Chondrocyte volume at day 1 of culture. Data were from [$N(n')=6(91)$ and $6(69)$] samples in stiff and soft gels respectively. **(b)** Percentage of chondrocytes with processes during culture in stiff or soft gels. Data were from [$N(n')=6(175)$, $6(111)$ at day 1 and $6(228)$, $6(491)$ at day 3] samples in stiff and soft gels respectively. **(c)** Number of processes per cell and **(d)** percentage of chondrocytes with processes of small, medium or long length categories at day 3 of culture in both gel strengths. Data were from [$N(n')=6(228)$ and $6(491)$] samples in stiff and soft gels respectively. * indicated a significant difference by unpaired Student's T-test, # indicated a significant difference according to one-way ANOVA. Double and triple symbols showed the level of significance for $P<0.01$ and 0.001 respectively.

Day 7 - Chondrocyte morphology

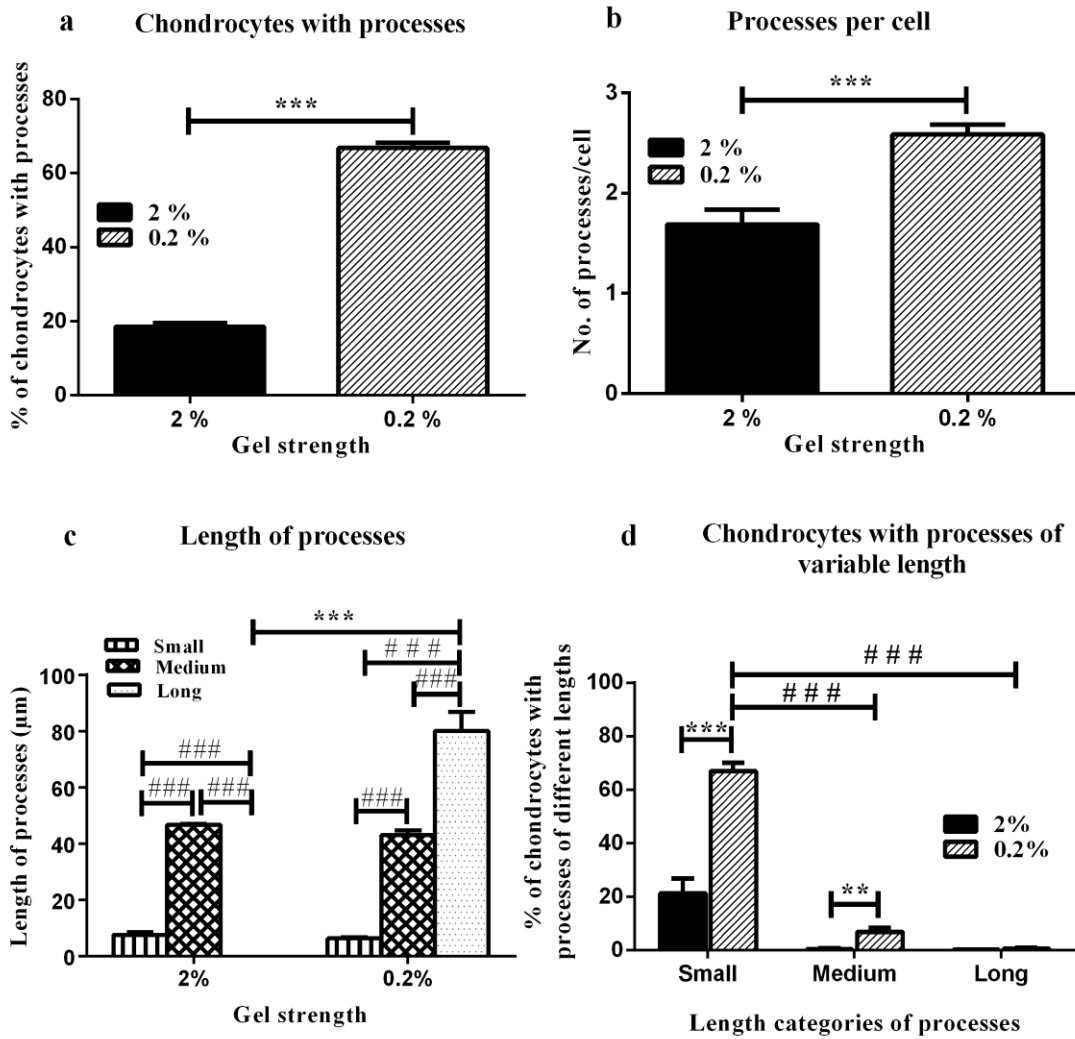


Fig. 3: Characteristics of abnormal chondrocyte morphology by day 7 of culture. Graphs displayed pooled data for **(a)** percentage of chondrocytes with processes, **(b)** average number of processes per cell, **(c)** average length of processes of various categories and **(d)** percentage of chondrocytes with processes of various length categories following culture in stiff or soft agarose gels. Data were from [$N(n')=6(313)$ and $6(525)$] samples in stiff and soft gels respectively. * indicated a significant difference according to an unpaired Student's T-test, # indicated a significant difference according to one-way ANOVA. Double and triple symbols showed the level of significance for $P<0.01$ and 0.001 respectively.

Day 7 - Cluster formation

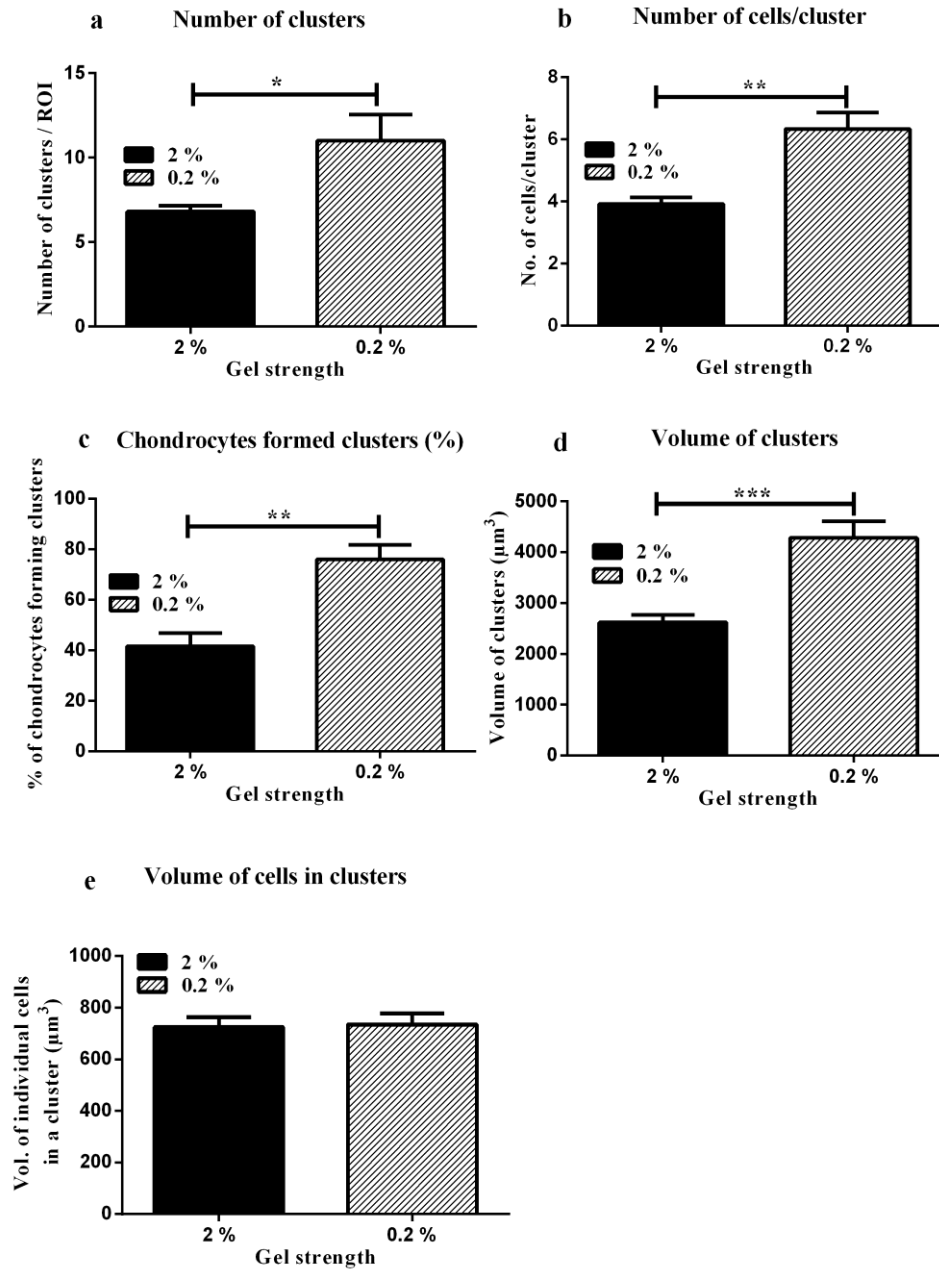


Fig. 4: Clusters of bovine chondrocytes formed following culture in stiff or soft agarose gels with 10% FCS by day 7. **(a)** Number of clusters, **(b)** number of cells per cluster, **(c)** percentage of chondrocytes forming clusters **(d)** volume of clusters (μm^3) and **(e)** volume of individual chondrocytes within clusters, following culture in stiff and soft agarose gels. Data obtained from $[N(n')]=[6(313)$ and $6(525)]$ in stiff and soft gels respectively. * indicated a significant difference according to an unpaired Student's T-test between stiff and soft gels. Single, double and triple symbols showed the level of significance for $P<0.05$, 0.01 and 0.001 respectively.

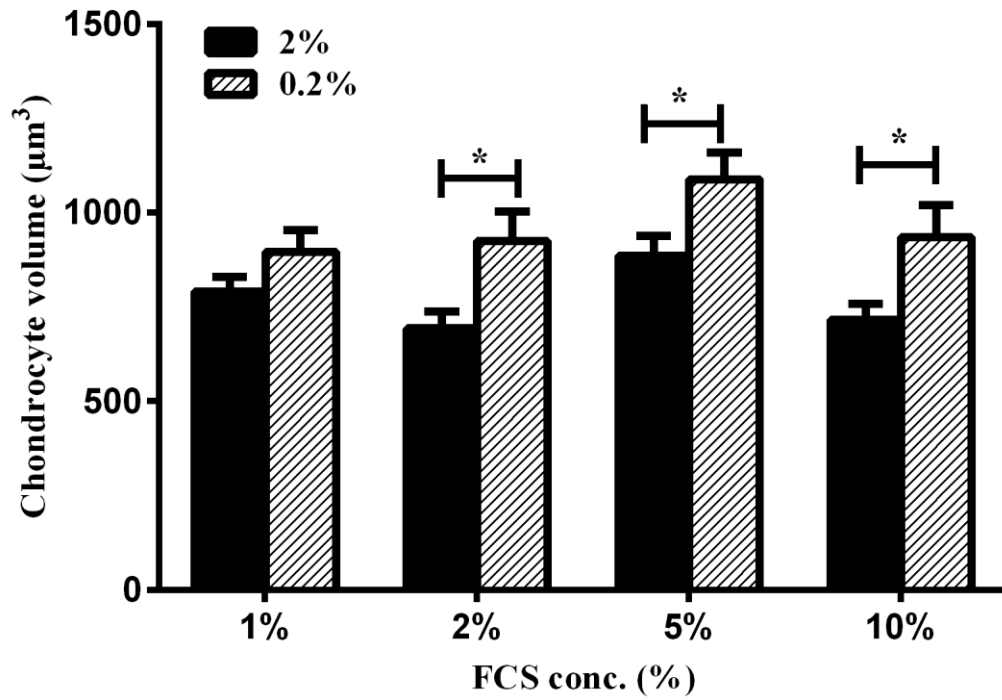


Fig. 5: Chondrocyte volume in stiff or soft agarose gels in the presence of increasing concentrations of FCS. The volume (in μm^3) of chondrocytes with normal morphology within agarose gels was shown with increasing serum levels. Data were from [$N(n')= 3(10, 6, 11, 4)$ in stiff gels and $3(16, 12, 5, 18)$] in soft gels cultured with 1%, 2%, 5% and 10% FCS concentrations respectively. * indicated a significant difference according to an unpaired Student's T-test with the level of significance for $P<0.05$.

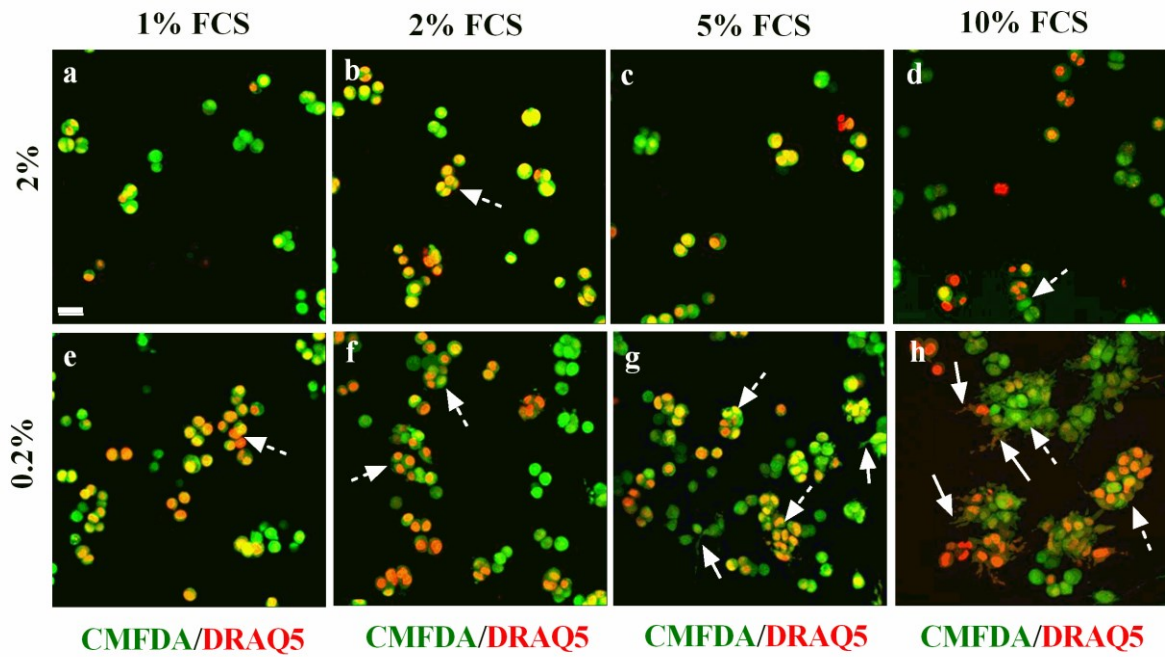


Fig. 6: Changes to chondrocyte morphology following culture in stiff or soft gels in the presence of increasing concentrations of FCS (1-10%). Axial CLSM high power (x40DW) magnification reconstructed images of CMFDA and DRAQ5-labelled chondrocytes (corresponding to the cytoplasmic space of live cells and DNA labelling respectively), at day 7 of culture in **(a,b,c,d)** stiff and **(e,f,g,h)** soft gels with **(a,e)** 1%, **(b,f)** 2%, **(c,g)** 5% and **(d,h)** 10% concentrations of FCS in the culture medium. Note the range of colours of living chondrocytes corresponding to the dual labelling of cells with the green and red fluorescence dyes (see Materials and Methods for further details). The solid arrows indicate examples of cytoplasmic processes and broken arrows indicate examples of cell clusters. Scale bar for all panels = 25 μ m.

Day 7 - Chondrocyte morphology

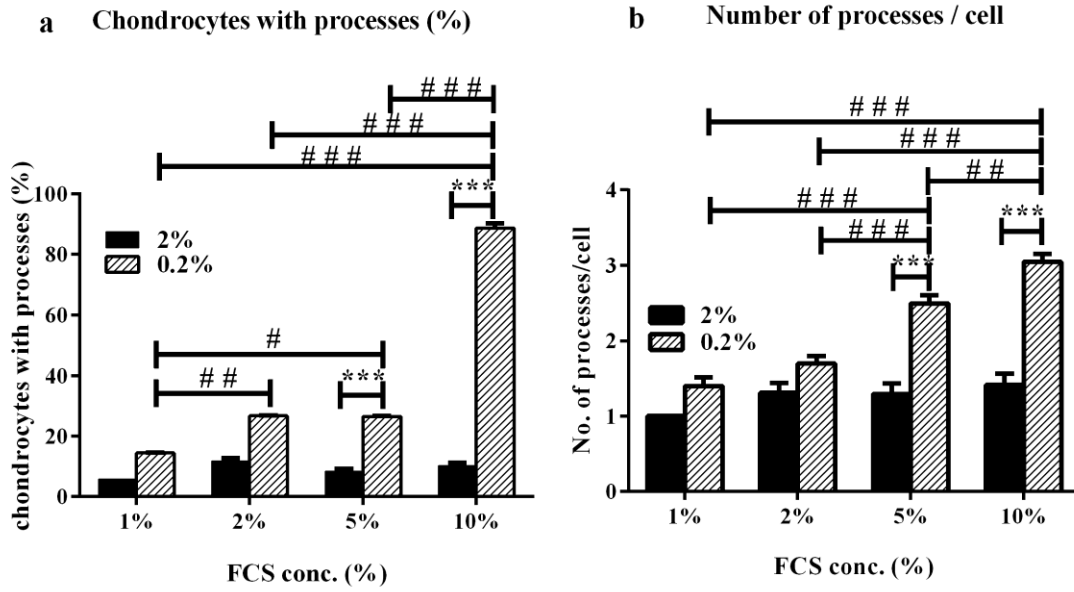


Fig. 7: Abnormal chondrocyte morphology following 7 days of culture in stiff or soft agarose gels with increasing concentrations of FCS. Graphs displayed data for the **(a)** percentage of chondrocytes with processes and **(b)** number of processes per cell. Data were from [$N(n)=6(35, 34, 180, 98)$ in stiff gels and $6(222, 334, 389, 569)$] in soft gels cultured with 1%, 2%, 5% and 10% concentrations of FCS respectively. * indicated a significant difference according to an unpaired Student's T-test between stiff and soft gels, # indicated a significant difference according to one-way ANOVA between soft (0.2%) gels. Single, double and triple symbols showed the level of significance for $P<0.05$, 0.01 and 0.001 respectively.

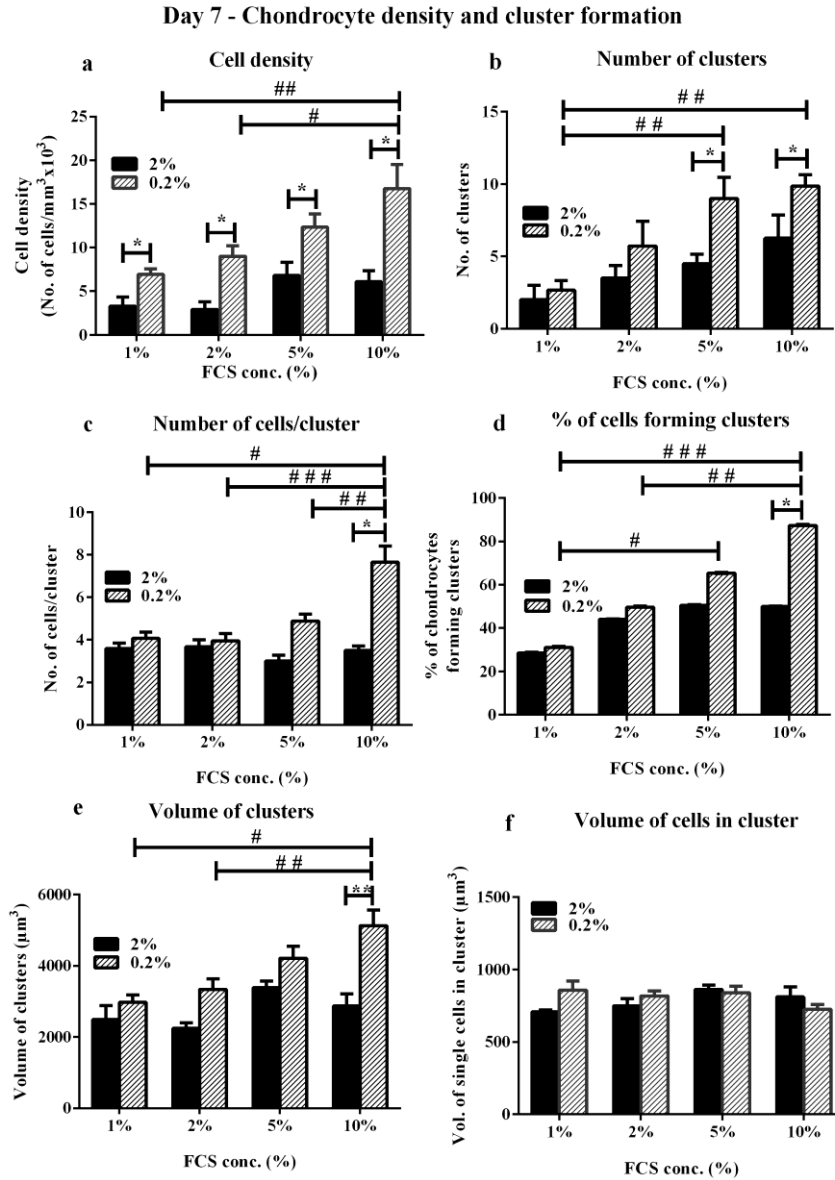


Fig. 8: Chondrocyte density and cluster formation by day 7 in stiff or soft gel cultures in the presence of increasing concentrations of FCS. Graphs showed data for **(a)** chondrocyte density (no. of cells per mm³ × 10³), **(b)** number of clusters, **(c)** number of cells per cluster, **(d)** percentage of chondrocytes forming clusters, **(e)** volume (μm³) of clusters and **(f)** volume (μm³) of individual cells in clusters. Data were obtained from [N(n')=6(35, 34, 180, 98) in stiff gels and 6(222, 334, 389, 569)] in soft gels cultured with 1%, 2%, 5% and 10% concentrations of FCS respectively. * indicated a significant difference according to an unpaired Student's T-test between stiff and soft gels, # indicated a significant difference according to one-way ANOVA between soft (0.2%) gels. Single, double and triple symbols showed the level of significance for P<0.05, 0.01 and 0.001 respectively.